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Intellectual Property Department
ZENECA Agrochemicals
Jealott's Hill Research Station
P O Box 3538
Bracknell Berkshire RG42 6YA
UNITED KINGDOM

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IMPROVEMENTS IN OR RELATING TO ORGANIC COMPOUNDS

The present invention relates *inter alia*, to polynucleotide sequences and their use in methods of providing herbicide resistance in plants. In particular the polynucleotides and methods may be used to confer resistance to herbicides comprising a chemical selected from the group consisting of: fomesafen; acifluorfen; chlorimuron ethyl and acetochlor.

Plants which are substantially "tolerant" to a herbicide when they are subjected to it provide a dose/response curve which is shifted to the right when compared with that provided by similarly subjected non tolerant like plants. Such dose/response curves have "dose" plotted on the x-axis and "percentage kill", "herbicidal effect" etc. plotted on the y-axis. Tolerant plants will typically require at least twice as much herbicide as non tolerant like plants in order to produce a given herbicidal effect. Plants which are substantially "resistant" to the herbicide exhibit few, if any, necrotic, lytic, chlorotic or other lesions when subjected to the herbicide at concentrations and rates which are typically employed by the agrochemical community to kill weeds in the field. Hereinafter the words (i) "tolerant" and (ii) "resistant" when used individually mean "tolerant and/or resistant".

Herbicide resistant plants are already available within the art for example, ROUNDUP READY™ Soya which is resistant to herbicides having as a site of action the enzyme 5-enolpyruvylshikimate-3-phosphate synthase, such as those agrochemicals containing glyphosate. One of the advantages of these plants is that the farmer can apply the herbicides to fields containing the resistant crop plants and weeds using "over-the-top application", to kill the weeds.

Other examples of herbicide resistant plants and products and methods for their production are shown in International Patent Application Publication Number WO 93/01294 and WO99/14337. Here the resistance is achieved by inserting into the plant a polynucleotide which provides for the production of a glutathione-S-transferase (GST) enzyme which is involved with the detoxification of the herbicide. Glutathione-S-transferase enzymes have been shown to exist in various organisms such as bacteria, fungi, yeast, plants, mammals and fish and may exist as homo or heterodimers with subunits typically between 24 and 30 KDa. It has been shown that herbicide detoxification is achieved by the conjugation of the herbicide with the free thiol glutathione (GSH), a tripeptide (gamma-

glutamyl-cysteinyl-glycine) within the plant (Cole D.J. 1994 Pesticide Science. 42 pp209-222). Such conjugation is catalysed by GST. Detoxification of herbicides has also been shown to occur following the conjugation of the herbicide with homoglutathione, which is the predominant thiol in some leguminous species. Homoglutathione (hGSH) is also a tripeptide (gamma-glutamyl-cysteinyl-Beta-alanine) but differs from GSH by the addition of Beta-alanine instead of a glycine to the gamma-glutamyl-cysteinyl part.

Thus, the present invention seeks to provide *inter alia*, novel polynucleotides which encode proteins which can be used in methods of providing herbicide resistant plants.

According to the present invention there is provided a protein comprising the amino acid sequence depicted as SEQ ID No. 1 or a protein variant having at least about 70% identity therewith wherein the said protein or variant is capable of catalysing the addition of Beta-alanine onto gamma glutamylcysteine. In particular the protein variant may be achieved through conservative substitutions within the amino acid sequence of the active protein which substitutions do not significantly adversely affect the activity of the variant protein. For example substitutions may be made between the following amino acid groups *viz.*

(a) Alanine, Serine, Glycine and Threonine

(b) Glutamic acid and Aspartic acid

(c) Arginine and Lysine

(d) Isoleucine, Leucine, Valine and Methionine

(e) Phenylalanine, Tyrosine and Tryptophan

It is particularly preferred that the protein variant has a Km for Beta-alanine which is less than the said protein variants Km for glycine when calculated using the same method. Preferably, the Km for Beta-alanine is less than or equal to about 10mM, more preferably 5mM, more preferably 4mM, more preferably 3mM, more preferably 2mM, more preferably 1mM and more preferably 0.8mM. The comparison of the Km with respect to Beta-alanine and glycine of the protein variants according to the present invention is calculated using the same method.

It is further preferred that the said protein variant comprises a region having the amino acid sequence depicted as SEQ ID No. 2 (KKIQQELAKP) and/or SEQ ID No. 3

(CFAGLWSL) and/or SEQ ID No. 4 (VMKPQREGGGNNIYG) and/or SEQ ID No. 5 (AAYILMQRIFP). The present invention still further provides a polynucleotide encoding the protein or protein variant referred to above and it is particularly preferred that the said polynucleotide comprises the sequence depicted as SEQ ID No. 6. Proteins and protein variants falling within this group may be referred to as homoglutathione synthetases.

In a further aspect of the present invention there is provided a Glutathione-S-transferase comprising the amino acid sequence depicted as SEQ ID No. 7 or 8 or 9 or 10 and a polynucleotide comprising a region which encodes at least one of the said amino acid sequences, preferably the sequence depicted as SEQ ID No. 11 or 12 or 13 or 14. The present invention also provides a polynucleotide sequence which is the complement of one which will hybridise to the polynucleotide of the preceding sentence under stringent conditions and which polynucleotide sequence still encodes a Glutathione-S-transferase. An example of such stringent conditions is hybridisation conducted at 65°C in a solution containing 6xSSC, 0.01% SDS and 0.25% skimmed milk powder, followed by washing at 65 °C in a solution containing 0.2xSSC and 0.1% SDS.

In a further aspect of the present invention there is provided a polynucleotide sequence which is the complement of one which binds to SEQ ID No. 11 or 12 or 13 or 14 at a temperature of between 60°C and 65°C in 0.3 strength citrate buffered saline containing 0.1% SDS followed by rinsing at the same temperature with 0.3 strength citrate buffered saline containing 0.1% SDS wherein said polynucleotide sequence still encodes a functional Glutathione-S-transferase enzyme with the *proviso* that the said polynucleotide sequence or the amino acid sequence which it encodes, is not a sequence selected from the group of sequences listed under accession number; P32110, U20809, Q03663, P32111, P46421, AJ000923, AF004358, AC000348 or AF051238. In particular the polynucleotide sequence of the preceding sentence is not a sequence selected from the group depicted as SEQ ID Nos. 25 to 34.

The present invention further provides a DNA construct comprising in sequence a plant operable promoter operably linked to a polynucleotide or polynucleotide sequence encoding a GST according to the present invention operably linked to a terminator region.

The present invention still further provides a DNA construct comprising in sequence a plant operable promoter operably linked to a polynucleotide or polynucleotide sequence encoding a protein or protein variant referred to above operably linked to a terminator region.

The present invention still further provides a DNA construct comprising a first region comprising in sequence a plant operable promoter operably linked to a polynucleotide encoding a protein or protein variant referred to above and a terminator region and a second region comprising in sequence a plant operable promoter operably linked to a polynucleotide or polynucleotide sequence encoding a GST according to the present invention and a terminator region. It is particularly preferred that the said first region contains a polynucleotide encoding the amino acid sequence depicted as SEQ ID No. 1 and the said second region contains a polynucleotide encoding the amino acid sequence depicted as SEQ ID No. 10. It is also preferred that the DNA construct referred to above further comprises a third region which provides for the production of a protein which acts as a selectable marker such as those that provide for antibiotic resistance e.g. kanamycin resistance or those providing for herbicide resistance e.g. glufosinate resistance.

It is preferred that the plant operable promoter within the DNA construct according to the present invention is constitutive, tissue specific, inducible or developmentally regulated. Such promoters, which are *per se* not germane to the invention, are well known to the skilled person and include, for example such as the constitutive CaMV35S, FMV35S, NOS, OCS, Patatin and E9 (derived from the small subunit of RUBISCO) or inducible such as the alcA/alcR gene switch described in published International Patent Application No. WO93/21334; the GST promoter switch described in published International Patent Application Nos. WO90/08826 and WO93/01294 and the RMS switch system described in published International Patent Application No. WO90/08830. Also the promoter may be developmentally regulated and/or tissue specific promoters for example the oleosin, ribulose bisphosphate carboxylase-oxygenase small sub-unit promoters. Terminators which can be used in the present invention include; Nos and the terminator of a gene of alpha-tubulin (EP-A 652,286).

The DNA construct of the present invention may further comprise (a) transcriptional enhancing elements; and/or (b) regions encoding non translated translational enhancing sequences, preferably Omega and Omega prime; and/or (c) regions encoding non translated

sequences such as intron sequences; and/or (d) regions encoding target sequences which are capable of directing transcription products to either intracellular organelles, intracellular compartments, cell membranes or to the outside of the cell.

In a further aspect of the present invention there is provided a method of providing plants which are resistant and/or tolerant to an agrochemical comprising (a) inserting into the genome of plant a polynucleotide or a polynucleotide sequence and/or a DNA construct which contains a GST according to the present invention; and (b) regenerating morphologically normal fertile plants or plant parts therefrom; and (c) applying to said plants or plant parts an amount of said agrochemical which is phytotoxic to control like plants and selecting those plants or plant parts which are resistant to said agrochemical.

In a further aspect of the present invention there is provided a method of providing plants which are resistant and/or tolerant to an agrochemical comprising (a) inserting into the genome of a plant which plant provides for the production of a functional Glutathione-S-transferase, a polynucleotide or a DNA construct comprising a protein or protein variant according to the present invention; and (b) regenerating morphologically normal fertile plants or plant parts therefrom; and (c) applying to said plants or plant parts an amount of said agrochemical which is phytotoxic to control like plants and selecting those plants or plant parts which are resistant to said agrochemical.

In a further aspect of the present invention there is provided a herbicide resistant plants obtainable by the method of the preceding paragraph.

In a further aspect of the present invention there is provided a method of providing plants which are resistant and/or tolerant to an agrochemical comprising (a) inserting into the genome of a plant a DNA construct comprising a first region comprising in sequence a plant operable promoter operably linked to a polynucleotide encoding a protein or protein variant referred to above and a terminator region and a second region comprising in sequence a plant operable promoter operably linked to a polynucleotide or polynucleotide sequence encoding a GST according to the present invention and a terminator region. It is particularly preferred that the said first region comprises a polynucleotide encoding the amino acid sequence depicted as SEQ ID No. 1 and the said second region comprises a polynucleotide encoding the amino acid sequence depicted as SEQ ID No. 10; and (b) regenerating morphologically normal fertile plants or plant parts therefrom; and (c) applying to said plants or plant parts an

amount of said agrochemical which is phytotoxic to control like plants and selecting those plants or plant parts which are resistant to said agrochemical.

In a further aspect of the present invention there is provided a herbicide resistant plants obtained by the method of the preceding paragraph.

It is particularly preferred that the agrochemical used in the methods described above comprises acifluorfen and/or chlorimuron-ethyl and/or fomesafen and/or acetochlor and/or metolachlor.

The polynucleotides and polynucleotide sequences and DNA constructs described above may be inserted into the genome of the selected plants using standard transformation techniques including particle mediated biolistic transformation, *Agrobacterium*-mediated transformation, protoplast transformation (optionally in the presence of polyethylene glycols); sonication of plant tissues, cells or protoplasts in a medium comprising the polynucleotide or vector; micro-insertion of the polynucleotide or vector into totipotent plant material (optionally employing the known silicon carbide "whiskers" technique), electroporation and the like.

In a further aspect of the present invention there is provided plants or plant parts obtained according to the methods described above. It is particularly preferred that the plants or plant parts are selected from the group consisting of: melons, mangoes, soybean, cotton, tobacco, sugarbeet, oilseed rape, canola, flax, sunflower, potato, tomato, alfalfa, lettuce, maize, wheat, sorghum, rye, bananas, barley, oat, turf grass, forage grass, sugar cane, pea, field bean, rice, pine, poplar, apple, peaches, grape, strawberries, carrot, lettuce, cabbage, onion, citrus or nut plants.

In a further aspect of the present invention there is provided the use of a polynucleotide or a polynucleotide sequence according or a DNA construct according to the present invention in a method of producing plants which are resistant and/or tolerant to a herbicide comprising acifluorfen and/or chlorimuron-ethyl and/or fomesafen and/or acetochlor and/or metolachlor.

In a further aspect of the present invention there is provided a method of selectively controlling weeds in a field said field comprising crop plants and weeds said method comprising applying to said field an agriculturally acceptable formulation of an agrochemical comprising acifluorfen and/or chlorimuron-ethyl and/or fomesafen and/or acetochlor and/or

metolachlor or a functionally related analogue thereof, characterised in that the said crop plants are the plants obtained according to the methods described above.

In a further aspect of the present invention there is provided the use of an agrochemical comprising acifluorfen and/or chlorimuron-ethyl and/or fomesafen and/or acetochlor and/or metolachlor or a functional analogue thereof to selectively control weeds in a field which field comprises crop plants and weeds comprising applying to said field an agriculturally acceptable formulation of said agrochemical in an amount which is sufficient to be phytotoxic to said weeds but not said crop plants characterised in that said crop plants are the plants obtained according to the methods described above.

In a further aspect of the present invention there is provided a protein comprising the sequence depicted as SEQ ID No. 7 or a protein variant having a Smith-Waterman score greater than 1094 in the SWISSPROT database calculated using the FASTA3 algorithm wherein the said protein variant still encodes a glutathione-S-transferase.

In a further aspect of the present invention there is provided a protein comprising the sequence depicted as SEQ ID No. 8 or a protein variant having a Smith-Waterman score greater than 619 in the SWISSPROT database calculated using the FASTA3 algorithm wherein the said protein variant still encodes a Glutathione-S-transferase.

In a further aspect of the present invention there is provided a protein comprising the sequence depicted as SEQ ID No. 9 or a protein having a Smith-Waterman score greater than 671 in the SWISSPROT database calculated using the FASTA3 algorithm wherein the said protein variant still encodes a Glutathione-S-transferase.

In a further aspect of the present invention there is provided a protein comprising the sequence depicted as SEQ ID No. 10 or a protein having a Smith-Waterman score greater than 766 in the SWISSPROT database calculated using the FASTA3 algorithm wherein the said protein variant still encodes a Glutathione-S-transferase.

The Fasta algorithm referred to above is well known to the skilled artisan and uses the method of Pearson and Lipman (Proc. Natl. Acad. Sci. USA 85; 2444-2448 (1988)) to search for similarities between one sequence and any group of sequences of the same type as the query sequence. Fasta also determines the best segment of similarity between the query sequence and the sequences in the database , using a variation of the Smith-Waterman algorithm. This "local alignment" procedure is described in Chao, Pearson, and Miller

(CABIOS 8; 481-487 (1992)). The score for this alignment is reported as the *opt* and Smith-Waterman score. The database used in the above calculations is the SWISSPROT database. This database, which is well known and frequently used by the person skilled in the art is commercially available from sources such as Geneva Bioinformatics (GeneBio™) S.A 25 avenue de Champel CH - 1206 Geneva Switzerland.

The present invention will now be described by way of the following non limiting examples with reference to the figures and sequence listing of which:

Figure 1. Vector pMJB2.

Figure 2. Vector pMOG800.

Figure 3. Vector pMOG1051.

Sequence Listing

SEQ ID No. 1. Homoglutathione synthetase from *Glycine max*.

SEQ ID Nos. 2 to 5. Homoglutathione synthetase protein fragments.

SEQ ID No. 6. Polynucleotide sequence encoding Homoglutathione synthetase from *Glycine max*.

SEQ ID Nos. 7 to 10. Glutathione-S-transferases 2.6, 3.1, 3.2 and 3.3 respectively.

SEQ ID No. 11 to 14. Polynucleotides encoding the GSTs 2.6, 3.1, 3.2 and 3.3 respectively.

SEQ ID Nos. 15 to 24. Primers.

SEQ ID No. 25 Soybean sequence P32110 derived nucleic acid sequence.

SEQ ID No. 26 Mungbean sequence U20809.

SEQ ID No. 27 Tobacco sequence Q03663.

SEQ ID No. 28 Potato sequence P32111 derived nucleic acid sequence.

SEQ ID No. 29 Arabidopsis sequence P46421.

SEQ ID No. 30 Arabidopsis sequence P46421 (genomic).

SEQ ID No. 31 Papaya sequence AJ000923.

SEQ ID No. 32 Spruce sequence AF051214.

SEQ ID No. 33 Wheat sequence AF004358.

SEQ ID No. 34 Spruce sequence AF051238.

Example

Production of plants with increased herbicide tolerance through increased *in planta* expression of (a) homoglutathione (b) homoglutathione and glutathione *S*-transferase (GST).

1.1 Isolation and preparation of polynucleotide sequence encoding hGSH synthetase.

Degenerate oligonucleotide primer MS-3 and OG9 are used in conjunction with PCR to amplify polynucleotide sequences encoding partial length Homoglutathione (hGSH) synthetase from first strand cDNA, synthesised using total RNA extracted from soybean cell cultures (cv. Mandarin) (Skipsey *et al.* 1997)

SEQ ID No. 15

MS-3 5' GCG AAG CCH CAR MGA GAR GGH GGA GG-3'

SEQ ID No. 16

OG-9 5' CGC ACT GAG AGA GGA TCC TCG AG 3'

The resulting PCR products are cloned into the vector pGEM-T (Promega™), which is termed MS3-1. The cloned insert is then excised from MS3-1 using *Eco* R1, labelled with digoxigenin using standard protocols (Boehringer Mannheim™), and used to screen a soybean cell culture cDNA library (cv. Mandarin), constructed in the uni-ZAP XR vector (Stratagene™).

1.2 Isolation and preparation of polynucleotide sequences encoding glutathione *S*-transferase

Degenerate oligonucleotide primers CJACON2 and CJACON3 are used independently in conjunction with oligonucleotide primer OG9 to amplify DNA probes from first strand cDNA suitable for cDNA library screening.

SEQ ID No. 17.

CJACON2 5' TTC TGG GYK RAS TWC ITY GAC RAI AAG 3'

SEQ ID No. 18.

CJACON3 5' GAG TCY MWK GTS ATT GTT GAA TAC ATT GAT GAG 3'

The PCR products obtained are cloned into the vector pCR 2.1 using the original TA cloning kit (Invitrogen™). Distinct cDNAs are identified by automated DNA sequencing (ABI 377) and labelled with digoxigenin in order to facilitate the screening of cDNA libraries for cDNAs encoding full length enzymes.

1.3 Preparation of constructs for use in heterologous gene expression in *E. coli*

Full length cDNAs encoding homoglutathione synthetase and glutathione *S*-transferase are independently expressed in *E.coli* using the pET expression system (Novagen™). *Nco* 1 and *Xho* 1 restriction enzyme sites are introduced by at the 5 prime and 3 prime ends of the homoglutahione synthetase cDNA respectively using primers MS4-*Nco* and MS-4-HIS.

SEQ ID No. 19

MS4-*Nco* 5' CCT CTC AAA CCC ATG GCT CAA CC 3'

SEQ ID No. 20

MS-4-His 5' GCG CTC GAG AGT TAG GTA TAC AGT ATC TAC C 3'

The PCR fragment is then digested with *Nco*1 and *Xho* 1 and ligated into similarly digested pET-24d. This vector is termed pET-MS4-His.

With respect to the cDNAs encoding glutathione *S*-transferase, either *Nde* I or *Nco* I sites are introduced as appropriate at the 5' end, and *Bam* H1 at the 3' end using PCR as described previously. The cDNA is then introduced into pET-24a or pET-24d as appropriate.

1.4 Expression and characterisation of recombinant enzymes in *E. coli*

Glutathione *S*-transferase

The vectors described in section 1.3, harbouring cDNAs encoding GSTs, are transformed into *E. coli* BL21 (DE3) using standard bacterial transformation procedures. Expression and

purification of the recombinant GST is performed according to the methods described by Cummins *et al.*, 1997.

Homoglutathione synthetase

Purification of homoglutathione synthetase is performed using the following method. *E. coli* BL21 (DE3) harbouring the pET-MS4-His plasmid (section 1.3) are grown at 30 °C until OD_{600}=0.5, after which isopropyl-β-D-thiogalactoside (IPTG) is added to a final concentration of 0.1 mM. Following a 3 hour incubation the bacteria are collected by centrifugation, re-suspended in buffer A (20mM Tris, 0.5 M NaCl, 5 mM imidazole pH 8.0) and lysed. Cell debris is removed by centrifugation (10,000 g, 10 min) and the supernatent applied to a 5 ml iminidiaceic acid column (Sigma™), previously charged with $NiSO_4$ and equilibrated in buffer A. The column is then washed buffer A containing 20 mM imidiazole, followed by buffer A containing 300 mM imidiazole to remove affinity bound protein. The protein in this fraction is then concentrated using a Centriplus 30 (Amicon™) spin column and re-suspended in buffer A prior to application onto a 1 ml HiTrap chelating column (Phamacia™), pre-charged with $NiSO_4$ and equilibrated as described previously. Affinity bound protein is recovered using an increasing concentration of 20-200 mM imidiazole and the presence of His-tagged recombinant homoglutathione synthetase detected using His-tagged antibodies according to standard procedures. Fractions containing recombinant homoglutathione synthetase are pooled, concentrated using Centricon 30 spin columns (Amicon™) and resuspended in 20 mM Tris-HCl pH 8.0, 1mM DTT.

1.5 Assay of enzyme activity of recombinant GST and homoglutathione synthetase.

GST assays are performed as described by Andrews *et al.*, 1997.

Homoglutathione synthetase is assayed for activity in 250 mM Tris-HCl pH 8.0, 50 mM KCl, 20 mM MgCl2, 5 mM DTT, 10 mM ATP, 1 mM γ-glutamylcysteine and 50 mM glycine or 10 mM β-alanine in a total 100 µl. Experimental controls consist of the exclusion of enzyme or glycine/β-alanine. Assays are performed at 30°C for 60 min, with 20 µl aliquots removed at regular time intervals. Monobromobimane derivatisation is then

performed on the aliquot to determine the presence of either glutathione or homoglutathione according to methods described by Cummins *et al.*, 1997.

1.6 Production of plant transformation vector harbouring cDNA encoding homoglutathione synthetase.

Oligonucleotide primers hGSH-Nco1 and hGSH-Kpn1 are used in conjunction with PCR to introduce *Nco*1 and *Kpn*1 sites at the 5 prime and 3 prime end of the cDNA respectively.

SEQ ID No. 21

Hgsh-Nco1 5' CCT CTC AAA CCC ATG GCT CAA CC 3'

SEQ ID No. 22

hGSH-Kpn 1 5' CGC GGT ACC TCC ATA CAA AGA AAA TCA 3'

The cDNA is then inserted into the vector pMJB2 (Figure 1) as a *Nco*1/*Kpn*1 fragment. The expression cassette, containing the CaMV35S double enhancer : Glucanase II leader, hGSH synthetase cDNA and *nos* terminator is the excised from pMJB2 using *Hind* III/ *Eco*R1 and ligated into the similarly digested binary vector pMOG800 (Figure 2).

1.7 Production of plant transformation vector harbouring tandem cDNAs encoding homoglutathione synthetase and GST.

Oligonucleotide primers 3.6-Bgl II and 3.6 *Nco* 1 are used in conjunction with PCR to introduce *Nco*1 and *Bgl* II sites at the 5 prime and 3 prime end of the GST 3.6 cDNA respectively.

SEQ. ID No. 23

3.6-Bgl II 5' GAG ATC TGC AAC AAA CAT AGC CTC 3'

SEQ ID No. 24

3.6 Nco 1 5' TAC ACC ATG GCT GAA AGG GAC TTG 3'

The GST cDNA is then inserted into the vector pMOG1051 (Figure 3) as a *Nco1/Bgl* II fragment. The expression cassette, containing the RolD/Fd promoter , GST cDNA and potato PI-II terminator is the excised from pMOG1051 using *Bam* H1 and ligated into the unique *Bam* H1 site in the binary vector containing the hGSH expression cassette (section 1.6). Orientation of the insert is determined by PCR.

1.8 Plant Transformation and Regeneration.

Constructs from section 1.6 and 1.7 are transformed into *Agrobacterium tumefaciens* strain LBA 4404 using the freeze thaw method of transformation provided by Holsters *et al.*, 1978. Tobacco transformation and whole plant regeneration is performed using var. Samusun according to protocols detailed by Bevan, 1984. Transformation events are selected on MS-media containing kanamycin.

1.9 Analysis of Transgenic Plants

PCR analysis of transformants

Leaf samples were taken from transformed lines and DNA extracted according to the methods described by Edwards et al., 1991. Oligonucleotide primers are designed to specific regions within the transgene to enable it's detection in the plant material tested.

RNA analysis

The presence of mRNA encoding the transgene is detected within the plant using Northern Blot hybridisation. Total RNA is extracted from leaf tissue using Tri-reagent and protocols provided by the manufacturer (Sigma™). Blots are prepared using existing procedures (Sambrook, 1989) and probed using radio-labelled cDNA encoding the desired mRNA.

Protein Analysis

The presence of recombinant protein in the transgenic plant is determined using Western blotting procedures with antibodies raised to the relevant enzyme using standard protocols.

Herbicide tolerance tests

Following tissue culture, transgenic plants are transferred to 5 inch pots containing John Innes potting compost no. 3. The plants are allowed to acclimatise (approx. 2 weeks) and various herbicides applied at various concentrations to the aerial tissue using a track sprayer. Visual assessment of phytotoxicity/plant necrosis is performed 6 and 13 days post application. Plants show resistance to herbicides applied at concentrations which are phytotoxic to control like plants.

1.10 Production of homozygous plant lines

Single copy transgenic plant lines are identified by Southern blot analysis according to methods described by Sambrook, 1989 using appropriate radiolabelled probes. Segregation analysis is performed on plants containing single insertion events by germination on MS media containing kanamycin. Further confirmation of homozygous lines is performed by back crossing transgenic lines with wild-type tobacco and analysis genetic segregation following selection on kanamycin.

References

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Cummins I., Moss S., Cole D.J and Edwards R (1997a) Glutathione Transferases in Herbicide-resistant and Herbicide-Susceptible Black-grass (*Alopecurus myosuroides*). *Pesticide Science* **51** 244-250.

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Holsters M., de Waele D., Depicker A., Messens E., van Montagu M and Schell J (1978) Transfection and transformation of *Agrobacterium tumefaciens. Molecular and General Genetics* **163** 181-187.

Skipsey M., Andrews CJ, Townson J.K., Jepson I and Edwards R (1997) Substrate and thiol specificity of a stress-inducible glutathione transferase from soybean. *FEBS Letters* **409** 370-374.

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gca 2763

<210> 26
<211> 1137
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Mungbean
Sequence U20809

<400> 26
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gttgaagaaa atttccgcaa caagagtgaa cagcttctga aatacaaccc agttcacaag 180
aaggttccag tgtttgttca tggtgacaaa ccccttccag agtcccttgt gattgttgag 240
tacatcgatg agacatggaa caacaacccc atcttggctt ctgatcctta ccagagagcc 300
ttggctcgtt tctggtccaa attcatcgat gacaagattg tgggtgcttc gtggaaatct 360
gttttcacag ttgatgagaa agagcgtgag aagaatattg cagaaacata tgagagtctg 420
cagtttcttg agaatgagat aaaggagaag aagttctttg gaggagaaga gcttgggttg 480

gtagatattg ctgctgtcta tgtagcattt tggatccctt tgattcaaga aatagcagga 540
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tataaggaaa attgtgtgga gtacttagtt aggatttggt ttcaaaatta tggttgaagt 780
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accaacaaga ttaagtaagg ctgcattatt tgtctttttg ttaaattaga tattagtatg 960
caccaaaaag tgagtatttc cttacagaag ctttttaaat attaagtagt taattccata 1020
ggtctaccat tatagctcaa gttatataca tattatgggt gccattctct actcaacaat 1080
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<210> 27
<211> 2038
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Tobacco
sequence Q03663

<400> 27
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aaaacgaaat aaacataagc tatttacaaa aaataaaaaa atactccatt tgaatctaaa 120
gtcaagtcgt gattgggata agaaaataga aatttattta tactccagat caagccgtga 180
ttggaatgag ataatagaaa agtatgatag tacatgagta acatcaagtt ggaaattaag 240
ggaaggaaat tagagaaaga actgaagaat atccaaatat tctttacgtc caaatttgat 300
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gaaagaagaa gacacaaact gtgtttggta ttattatagt tttttctttt agagaattga 420
ttgtacatat aagaaatata atataagatt tagaaataag attattagaa aaatcaaaca 480
tcaaagtatt tattttaaat tctttttcca atggacattc ccattctgaa aaaaaagaga 540
tataaatatg gaagtaaaaa ttaatcagat cgttaaatgt agaaaatatt aattaacaca 600
ttaaccataa ccagtctact ttatttaaca aaaagcacat ctgaratarc aaaaaagtgt 660
ttaacttcat gcattgacaa tttaaaatta ttttgcaaca tcgggtaaaa ctattttaca 720
acaattggta actgcatata taagtttaat atggtaacct agaaaatagg ataaattatc 780
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taggtaaata tttatgacga attctcaata gtaatctgaa aaaaaattgt aactaaccta 1980
ttatactaaa actactataa taggttagat tacattaatc atgtcattag aagatctt 2038

<210> 28
<211> 2796
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Potato- Derived
nucleic acid sequence P32111

<400> 28
aagcttacat tcaacgtgtt gctgcttcaa aataagggtc tttacaaaat ttaaaaaata 60
taaagaagaa ttttgaattt tataattaaa gccgtcagaa aggacttgac ctttgaagcc 120
aacccaactc aatattagaa aatcaaaaat tttagtgcat tcatttataa aaaaaaaaaa 180
aattacttat gcagttcttg aacccttttgt gagacgagag ggagttgctc ggatggtaag 240
cacccttcac tttcaacccg aaggttgcga gtttgagtca ccaacggagc aaaaagggta 300
ggagctccta gaaagggtaa aaaaaaaaaa aaaaattaat aaaaaaatac cctttatgaa 360
atttctcatt ccgctactgc acttctcccc tgatcttcct cgtgttttca attattaatt 420
ctatattcat gacaccatgt gatgtttctc tgggtagtcc taaaaataga ggtattgaaa 480
attatgttgt ttctctctgg tctatttact tttcttgtgt actttattgt atttcatatt 540
gttaattttt ggcttcgttt tataacatgt tattagcaca aaactttaat catatcgagt 600
taaactttta attttgctta tcaacgtaaa agacaagata tgtgatcggc atgtataact 660
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tgtcgtttaa ctttggagcg tacaagtgtc tacttgtgat ccagtaggtt atcaaagctg 780
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aactccattc attttaattt attgtcatgt tttgacttga tccaaaatct aacaatttaa 1140

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tgaaatatat taaagttgca tgttataaga tttatcttta tttcactagt taatataaat 2760
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<210> 29
<211> 1289
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Arabidopsis
P46421

<400> 29
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aacatctgtt tccttctaga ctcttcgcat ttacatcaca ctgccgacca tataaaacgg 180
caaagttcgt cgtcgtttta tcacaagacc atcaacacca taaggctata aatccaagct 240

aaaaggtagt gattaactcc acaaaaccag aaaaactaca tttctaacat atagaagaaa 300
cagagaaaaa gagagagaga cccctaatgg ctgagaaaga agaagtgaag cttttgggga 360
tatgggcgag ccctttttagc cgtcgggtcg agatggctct caaactcaaa ggcataccgt 420
acgagtacgt ggaagagata ctggagaaca aaagcccttt gcttcttgct cttaacccta 480
ttcacaagaa agtccctgtt cttgtccaca atggtaaaac cattctcgag tctcatgtga 540
ttcttgaata catcgatgag acttggccac aaaatccaat tctccctcaa gatccttatg 600
aaagatccaa agctcgtttc tttgctaaac tcgtcgatga acaggtaatt gaattggttc 660
aaaattgcat gtcaaataat aaacaattgg ttctgctttg ttaatttatc aaacaagtaa 720
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gttgtgtggt tgagaagtga ggttttatca tcatctctca cgttatctta tttggtccca 1260
gccactattt agaattaatg gtaaagctt 1289

<210> 30
<211> 1339
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Arabidopsis
Genomic sequence

<400> 30
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acacatgctc cagggataag gcaaggttag gaattaggac acatctccac ttactaaaaa 120
agagataaaa aaaaattgta tagggaacgt tataaatatg ttgtaaagtc aacatctgtt 180
tccttctaga ctcttcgcat ttacatcaca ctgccgacca tataaaacgg caaagttcgt 240
cgtcgtttta tcacaagacc atcaacacca taaggctata aatccaagct aaaaggtagt 300
gattaactcc acaaaaccag aaaaactaca tttctaacat atagaagaaa cagagaaaaa 360
gagagagaga cccctaatgg ctgagaaaga agaagtgaag cttttgggga tatgggcgag 420
ccctttttagc cgtcgggtcg agatggctct caaactcaaa ggcataccgt acgagtacgt 480
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gtcaaataat aaacaattgg ttctgctttg ttaatttatc aaacaagtaa ttttctatta 780
acattagcga ttatatgtct ctgtcattgt agattatgaa cgtggggttt atatcaatgg 840

caagagcaga cgagaaagga agagaagttt tagccgagca ggtaagagaa ctgattatgt 900
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tggaagtgat tacagaggag aagtttccag agttcaagag atgggttagg aatttggaga 1080
aggttgagat tgttaaagat tgtgttccac caagagagga acatgtagaa cacatgaact 1140
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caatgtttgt atggttatgt tgttgtttat tttattgaat atctttgtat gttgtgtggt 1260
tgagaagtga ggttttatca tcatctctca cgttatctta tttggtccca gccactattt 1320
agaattaatg gtaaagctt 1339

<210> 31
<211> 968
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Papaya AJ000923

<400> 31
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gctccgtttt acaacaatgg cggacgaggt tgttctcttg gatttctggc caagcccttt 120
tggaatgaga atcagaatcg ctttagccga gaagggtatt cactacgagt acaaggaaga 180
gaatctgaga aacaagagtc ccttactcct gcagatgaac ccggtacaca agaaaatccc 240
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tgaggtatgg agcgacaagg ctcctctgct tccctctgat ccttatcaga gagctcaagc 360
caggttctgg gctgactatg ttgacaagaa gatgtatgaa gctgggagga gagtttggac 420
gactaaaggg gaagaacagg agggggccaa gaaagagttc atagaaatct tgaagacttt 480
ggagggagaa cttggggaga agccttattt tggtggggaa agttttgggt atgtggattt 540
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tcatatgt 968

<210> 32
<211> 1040
<212> DNA
<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:Spruce AF051214

<400> 32

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tgctgcgggt ccgtatagca cttgctctta aagggatcga ttacgagttc atcgaagaga 180
acatgcaaaa taagagccat ctgctgctgc aatcgaaccc tgttaacaag aagattccag 240
tgctcatcca caatggaaag cctgtttgtg agtccatgat tattgtgcag tacatagacg 300
aggcatggga cacgaaggcg ccagttctaa tgcccaaaga tccttatgac cgagccattg 360
cccgcttctg ggctgccttt gtagacgata agctgctgcc atgccttcgg ggagttttca 420
agggccaggg agagcagcaa cagaaagcgt tggaagaatc gggggcaagc tttcttttac 480
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aggttactaa tttagtttta atagaccagg agaagatgcc gctgttaagc acatggatga 660
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aatttatatc cgccatcaga gtcagattta catcaccacc tgctgccaat taggggaagc 780
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gaatgttgtt atgctttttg aattgttggt gtttaatggg aataattcta tcgcccaatc 900
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tttgggagat agaacaaaaa ttcatggaaa tgtgtgttag tgttttatat ttgaaaaggg 1020
ttggatttgc agagaatgga 1040

<210> 33
<211> 902
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Wheat AF004358.

<400> 33

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acactgacac acatcgatcg aggtagttag agatggccgg aggagatgac ctgaagctgc 120
tcggcgcatg gccaagccca tttgttacca gggtgaagct ggcgctcgcc ctgaagggcc 180
tgagctacga ggacgtggag gaggacctgt acaagaagag tgagcttctc ctcaagtcca 240
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tgatcattct ccagtacatc gacgaagtgt tcgccagcac cggcccgtcc cttcttccag 360
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tagccccatg gaggcagtgg ttgaggggca agacagagga ggagaaatcc gagggaaaga 480
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cgtggatgaa ggtgaccgag gcgctgtctg gtgacaagat tttcgacgcc gccaagactc 660
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aa 902

<210> 34
<211> 1127
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Spruce AF051238

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gacggaggct tgtggagaaa aggggcaagt gcagctgtta ggtgggagtc tgagttcctt 120
cgtgctgcgg gttcgcatag cacttgctct taaaggcatc gattacgagt tcatcgaaga 180
gaacatgcaa aataggagcc agctgctgct gcaatcaaac cctgttcaca ggaagattcc 240
agtgcttatc cacaatggaa agcccgtttg tgaatccatg attattgtgc agtacatcga 300
tgaggcatgg gacactaagg cacccaatct tatgcccaaa gatccatatg acctagccat 360
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cgctggccat ggcgagcagc tacagaaaga agcggaagat ctggttacaa actttcattt 480
gatagaggaa gctctgcgaa ccaacagctg cttctcagga aaagcgtatt ttggagggga 540
taagataggc ttgcttgaca tcgcattggg tggtatgttg gcggttctca aaggcctcga 600
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ggaatctcta tcagctagga gagccagact tgcatcacct gctggcaatt agggcaagcc 780
atgtcggcct tataaactga ggatagacag atggattata aacttattat tcgtagtact 840
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tacgctgatt tagtttgaac aagttttggt gaatctcccc tgtactgaag ctggcattcc 1020
cctgttcaat gtgcgctgat ttagtttgaa taagtttttg atgaatctcg cttgtactgt 1080
agctatgtgc gatgattttt aatgccatag aaacgagaat gaaatgc 1127

CLAIMS

1. A protein comprising the amino acid sequence depicted as SEQ ID No. 1 or a protein variant having at least about 70% identity therewith wherein the said protein or variant is capable of catalysing the addition of Beta-alanine onto gamma glutamylcysteine.

2. A protein variant according to claim 1 having a Km for Beta-alanine which is less than the said variants Km for glycine when calculated using the same method.

3. A protein variant according to claim 2 having a Km for Beta-alanine which is less than or equal to about 0.8mM and a Km for glycine which is higher than 0.8mM when calculated using the same method.

4. A protein variant according to claim 2 or 3 which variant comprises an amino acid sequence selected from the group depicted as SEQ ID No. 2, 3, 4 or 5.

5. A polynucleotide comprising a region encoding the protein or protein variant according to any one of claims 1 to 4.

6. A polynucleotide according to claim 5 which comprises the sequence depicted as SEQ ID No. 6.

7. A Glutathione-S-transferase comprising the amino acid sequence depicted as SEQ ID No. 7, 8, 9 or 10.

8. A polynucleotide comprising a region which encodes at least one of the amino acid sequences according to claim 7.

9. A polynucleotide sequence which is the complement of one which will hybridise to the polynucleotide according to claim 8 under stringent conditions and which polynucleotide sequence still encodes a Glutathione S transferase.

10. A polynucleotide according to claim 9 which comprises the sequence depicted as SEQ ID No. 11 or 12 or 13 or 14.

11. A polynucleotide sequence which is the complement of one which binds to SEQ ID No. 11 or 12 or 13 or 14 at a temperature of between 60°C and 65°C in 0.3 strength citrate buffered saline containing 0.1% SDS followed by rinsing at the same temperature with 0.3 strength citrate buffered saline containing 0.1% SDS wherein said polynucleotide sequence still encodes a functional Glutathione S transferase enzyme with the *proviso* that the said polynucleotide sequence or the amino acid sequence which it encodes, is not a sequence selected from the group of sequences listed under accession number; P32110, U20809, Q03663, P32111, P46421, AJ000923, AF004358, AC000348 or AF051238.

12. A DNA construct comprising in sequence a plant operable promoter operably linked to a polynucleotide according to either of claims 5 or 6 operably linked to a terminator region.

13. A DNA construct comprising in sequence a plant operable promoter operably linked to a polynucleotide or polynucleotide sequence according to any one of claims 8 to 11, operably linked to a terminator region.

14. A DNA construct comprising a first region comprising in sequence a plant operable promoter operably linked to a polynucleotide according to claim 5 or claim 6 and a terminator region and a second region comprising in sequence a plant operable promoter operably linked to a polynucleotide or polynucleotide sequence according to any one of claims 8 to 11 and a terminator region.

15. A DNA construct according to claim 14 wherein the said first region comprises a polynucleotide encoding the amino acid sequence depicted as SEQ ID No. 1 and the second region comprises a polynucleotide encoding the amino acid sequence depicted as SEQ ID No. 10.

16. A DNA construct according to any one of claims 12 to 15 which further comprises a third region which provides for the production of a protein which acts as a selectable marker.

17. A DNA construct according to any one of claims 12 to 16 wherein the plant operable promoter is selected from the group consisting of: CaMV35S, FMV35S, NOS, OCS, Patatin, E9, alcA/alcR switch, GST switch, RMS switch, oleosin, ribulose bisphosphate carboxylase-oxygenase small sub-unit promoters.

18. A method of providing plants which are resistant and/or tolerant to an agrochemical comprising:
(a) inserting into the genome of plant a polynucleotide or a polynucleotide sequence according to any one of claims 8 to 11 or a DNA construct according to any one of claims 13 to 17; and
(b) regenerating morphologically normal fertile plants or plant parts therefrom; and
(c) applying to said plants or plant parts an amount of said agrochemical which is phytotoxic to control like plants and selecting those plants or plant parts which are resistant to said agrochemical.

19. A method of providing plants which are resistant and/or tolerant to an agrochemical comprising:
(a) inserting into the genome of a plant which plant provides for the production of a functional Glutathione S transferase, a polynucleotide according to either of claims 5 or 6 or a DNA construct according to claim 12; and
(b) regenerating morphologically normal fertile plants or plant parts therefrom; and

(c) applying to said plants or plant parts an amount of said agrochemical which is phytotoxic to control like plants and selecting those plants or plant parts which are resistant to said agrochemical.

20. A method according to claim 18 or 19 wherein the said agrochemical comprises acifluorfen and/or chlorimuron-ethyl and/or fomesafen and/or acetochlor and/or metolachlor.

21. Plants or plant parts obtained according to the method of any one of claims 18 to 20.

22. Plants or plant parts according to claim 21 wherein said plants or plant parts are selected from the group consisting of: melons, mangoes, soybean, cotton, tobacco, sugarbeet, oilseed rape, canola, flax, sunflower, potato, tomato, alfalfa, lettuce, maize, wheat, sorghum, rye, bananas, barley, oat, turf grass, forage grass, sugar cane, pea, field bean, rice, pine, poplar, apple, peaches, grape, strawberries, carrot, lettuce, cabbage, onion, citrus or nut plants.

23. Use of a polynucleotide according to any one of claims 5,6, 8 or 10 or a polynucleotide sequence according to claim 9 or 11 or a DNA construct according to any one of claims 12 to 17 in a method of producing plants which are resistant and/or tolerant to a herbicide comprising acifluorfen and/or chlorimuron-ethyl and/or fomesafen and/or acetochlor and/or metolachlor.

24. Herbicide resistant plants obtained by the method of any one of claims 18 to 20.

25. A method of selectively controlling weeds in a field said field comprising crop plants and weeds said method comprising applying to said field an agriculturally acceptable formulation of an agrochemical comprising acifluorfen and/or chlorimuron-ethyl and/or formasafen and/or acetochlor and/or metolachlor or a functionally related analogue thereof, characterised in that the said crop plants are the plants according to claim 21, 22 or 24.

26. Use of an agrochemical comprising acifluorfen and/or chlorimuron-ethyl and/or fomesafen and/or acetochlor and/or metolachlor or a functional analogue thereof to selectively control weeds in a field which field comprises crop plants and weeds comprising applying to said field an agriculturally acceptable formulation of said agrochemical in an amount which is sufficient to be phytotoxic to said weeds but not said crop plants characterised in that said crop plants are the plants according to claim 21, 22 or 24.

27. A protein comprising the sequence depicted as SEQ ID No. 7 or a protein variant having a Smith-Waterman score greater than 1094 in the SWISSPROT database calculated using the FASTA3 algorithm wherein the said protein variant still encodes a Glutathione-S-transferase.

28. A protein comprising the sequence depicted as SEQ ID No. 8 or a protein variant having a Smith-Waterman score greater than 619 in the SWISSPROT database calculated using the FASTA3 algorithm wherein the said protein variant still encodes a Glutathione-S-transferase.

29. A protein comprising the sequence depicted as SEQ ID No. 9 or a protein variant having a Smith-Waterman score greater than 671 in the SWISSPROT database calculated using the FASTA3 algorithm wherein the said protein variant still encodes a Glutathione-S-transferase.

30. A protein comprising the sequence depicted as SEQ ID No. 10 or a protein variant having a Smith-Waterman score greater than 766 in the SWISSPROT database calculated using the FASTA3 algorithm wherein the said protein variant still encodes a Glutathione-S-transferase.

FIGURE 1

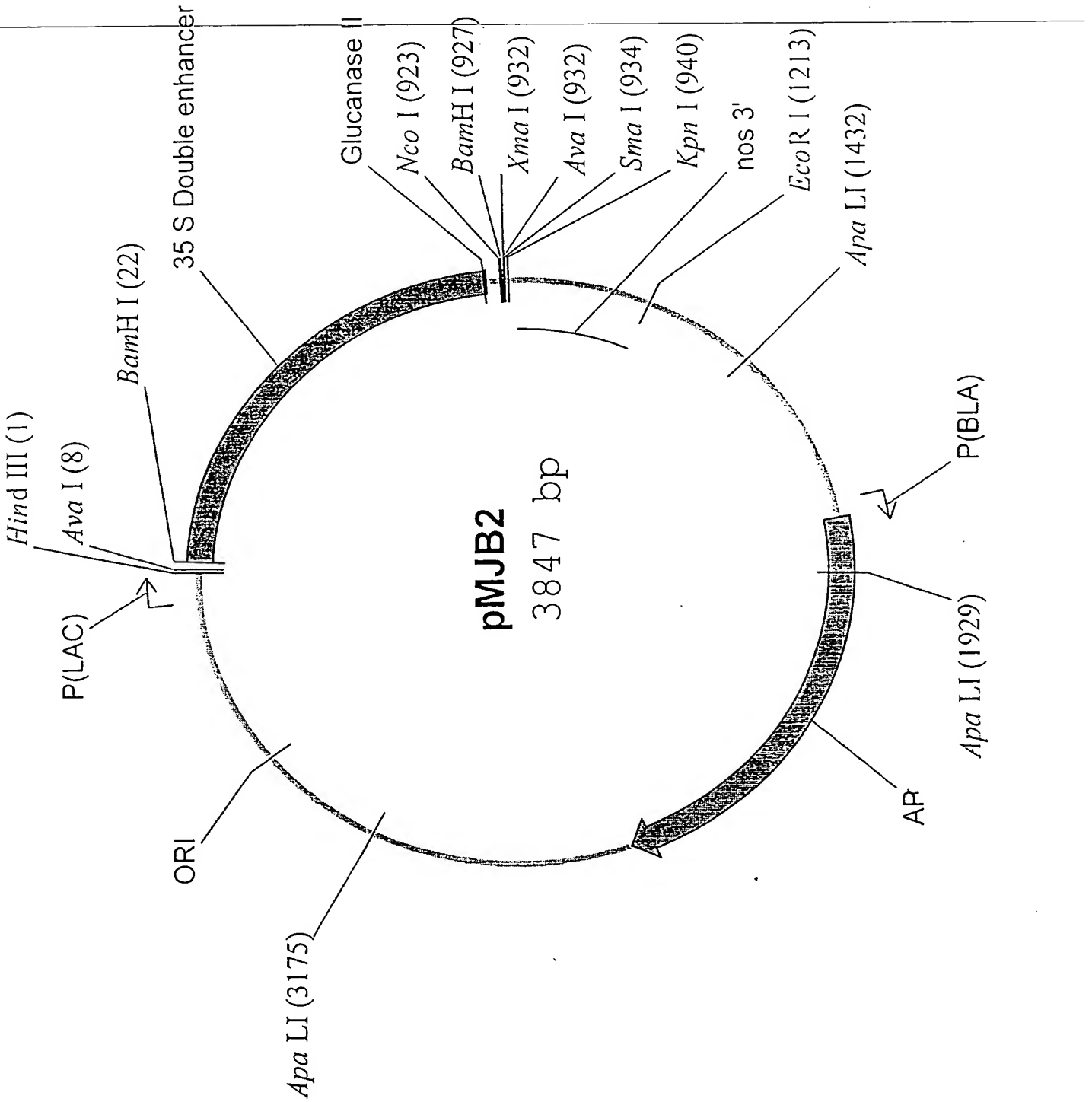

pMJB2
3847 bp
35 S Double enhancer
Glucanase II
Nco I (923)
BamH I (927)
Xma I (932)
Ava I (932)
Sma I (934)
Kpn I (940)
nos 3'
EcoR I (1213)
Apa LI (1432)
P(BLA)
Apa LI (1929)
AR
Apa LI (3175)
ORI
P(LAC)
Hind III (1)
Ava I (8)
BamH I (22)

FIGURE 2

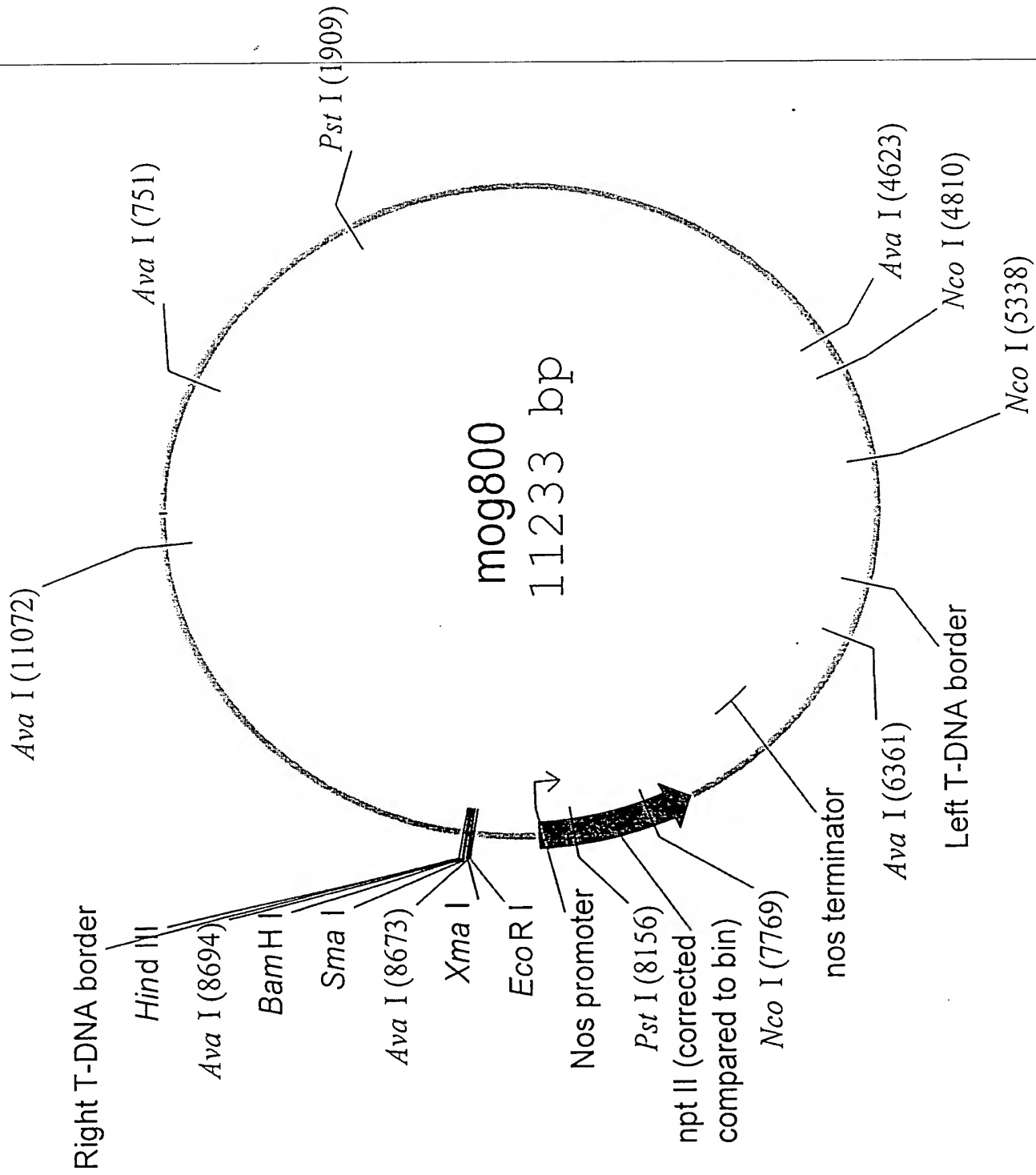
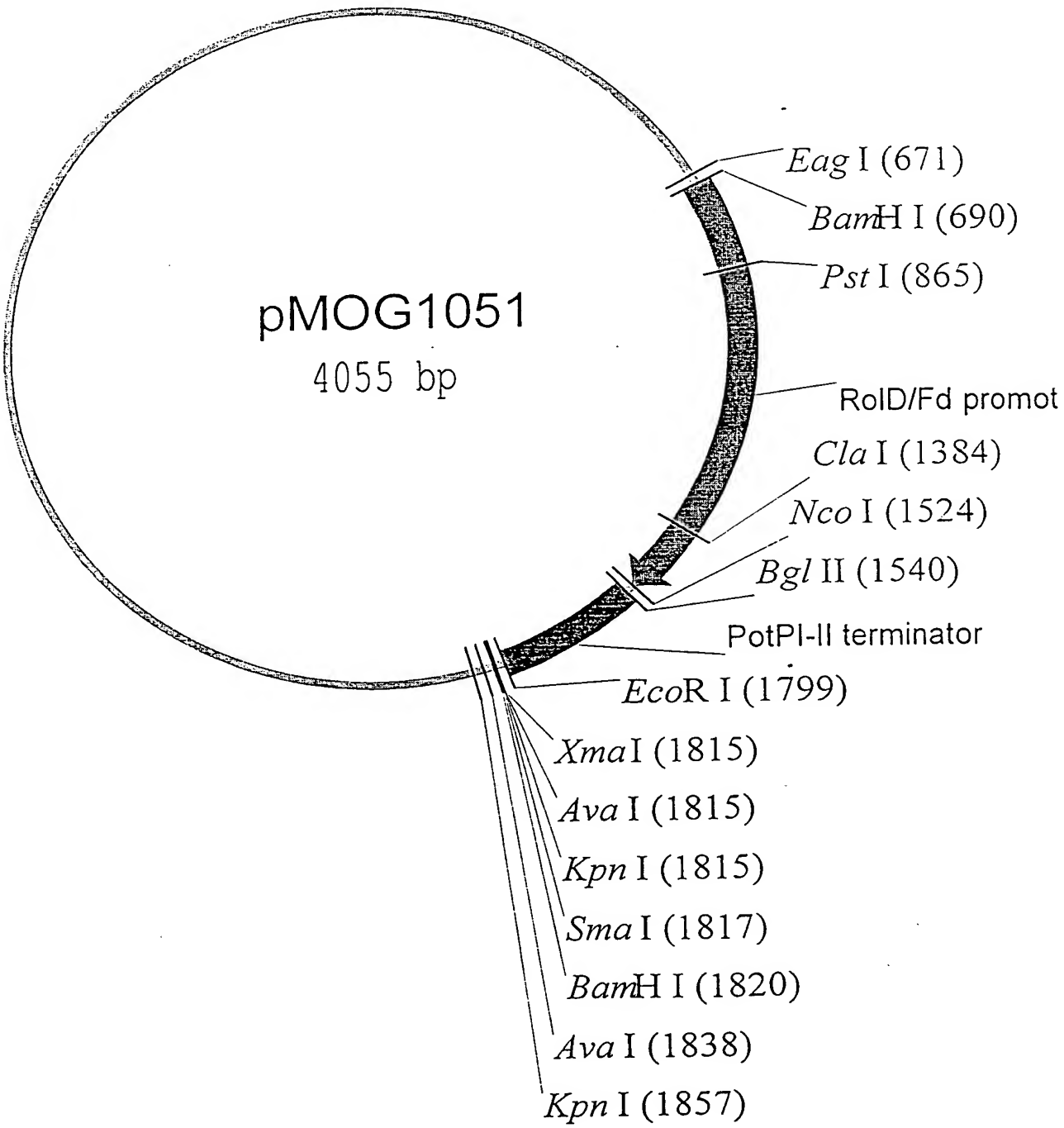

mog800
11233 bp
Pst I (1909)
Ava I (751)
Ava I (11072)
Ava I (4623)
Nco I (4810)
Nco I (5338)
Ava I (6361)
Left T-DNA border
nos terminator
Nco I (7769)
Pst I (8156)
npt II (corrected compared to bin)
Nos promoter
EcoR I
Xma I
Ava I (8673)
Sma I
BamH I
Ava I (8694)
Hind III
Right T-DNA border

FIGURE 3


pMOG1051
4055 bp
Eag I (671)
BamH I (690)
Pst I (865)
RolD/Fd promot
Cla I (1384)
Nco I (1524)
Bgl II (1540)
PotPI-II terminator
EcoR I (1799)
Xma I (1815)
Ava I (1815)
Kpn I (1815)
Sma I (1817)
BamH I (1820)
Ava I (1838)
Kpn I (1857)